BIOLOGIJA. 2005. Nr. 3. P. 59–61

Polyphenoloxidase isozyme and *Vfa1* sequence specific markers in apple cultivars differing in scab resistance

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The objective of the study was to elucidate the isozyme markers related to scab resistance. Polymorphism in the polyphenoloxidase (PPO, EC 1.14.18.1) enzyme system was investigated and DNA analysis using a *Vfa1* sequence specific marker was performed to identify the presence of *Vfa1* gene like sequences in apple cultivars having the above-mentioned isoforms. The number of PPO bands was found to change during the vegetation period. The PPO enzyme systems showed considerable variation among the apple cultivars. Some specific bands or their combination were determined for scab-susceptible and scab-immune apple cultivars. Our results showed that the cultivars 'Prima', 'Aldas', 'Skaistis' and 'Rudenis', which are immune to 1–5 *Venturia ineaqualis* races, have a DNA fragment of 500bp specific for the *Vfa1* gene. The immune cultivar 'Štaris' doesn't have this fragment. However, this fragment was detected in the genome of the cultivar 'Tellissare' having a polygenic scab resistance mechanism. The above-mentioned DNA fragment was not obtained for the susceptible cultivars 'Noris' and 'Papirovka' and the cultivars 'Katja' and 'Auksis' resistant to polygenic scab. The possible genetic background of such results is discussed.

Key words: apple cultivars, isozymes, scab resistance, DNA, *Vfa1*, PPO

INTRODUCTION

Apple scab is one of the most harmful apple diseases. Several sources of apple scab resistance have been identified and used in breeding programs, but mainly resistance determined by the *Vf* gene derived from *Malus floribunda* 821 was used [1, 2]. The initial *V* refers to *Venturia*, the genetic name of the scab incitant, and the subscript *f* refers to *M. floribunda* as a source of scab resistance germplasm [1]. Molecular markers have a very good potential for plant breeders. Particularly they is important for selecting trees that combine two or more genes for scab resistance, because traditional methods can be inefficient. A high level of isozyme polymorphism has been detected in apple and more than 20 polymorphic isozyme loci were identified [3, 4]. Polyphenoloxidase (PPO) is involved in the disease and pest resistance of trees [5, 6]. A cluster of four resistance paralogs (*Vfa1, Vfa2, Vfa3* and *Vfa4*) was identified in the *Vf* locus [7]. *Vfa1* has no introns and is predicted to endcode proteins characterized with extracellular leucine-rich repeats and transmembrane domains.

The objective of our work was to establish the isoforms of polyphenoloxidase related to apple scab resistance and identify the presence of *Vfa1* gene-like sequences in apple cultivars having the above-mentioned isoforms.

MATERIALS AND METHODS

The apple cultivars studied differ in their resistance to scab: 'Noris', 'Papirovka' are scab-susceptible, 'Antonovka', 'Katja', 'Tellissaare' and 'Auksis' show a good field resistance to scab, and 'Prima', 'Štaris', 'Aldas', 'Skaistis', 'Rudenis' are immune to scab. For determination of polyphenol oxidase (PPO, EC 1.14.18.1) polymorphism, leaves of the apple cultivars were collected during the growing period (May, July, September) from trees growing in an orchard. Leaf samples (0.5 g) were homogenized in Tris-Glyc buffer (pH 8.3) and electrophoresis was performed in vertical polyacrylamide gels [8]. After electrophoresis the gels were stained in a solution of L-3,4-dihydroxyphenylalanine and p-phenylenediamine, ac-

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cording to Jaaska [9]. The relative mobility (Rf) of polyphenol oxidase (PPO) isozyme bands was determined [9] from the average value of at least 4–10 repeats. The change of band number was estimated during the vegetation period.

Genomic DNA was extracted from leaf material using the minipreparation method as described by Dellaporta et al. [10]. For polymerase chain reaction (PCR) 1 unit of *Taq* DNA polymerase (MBI Fermentas, Lithuania), 1.5 mM $MgSO_4$, 0.2 mM dNTP and 1 μM of each oligonucleotide primer were used. DNA denaturation was performed at 95 °C for 4 min, with the further 35 cycles at 94 °C for 1.15 min, 47 °C for 1.15 min and 72 °C for 2 min.

The primers used, according Xu and Korban [7], were as follows:

For: 5'-TCTATCTCAGTAGTTTCTATAATTCC-3',

Rev: 5'-GTAGTTACTCTCAAGATTAAGAACTT-3'.

RESULTS

The number of PPO bands changed during the vegetation period of the apple cultivars (Table 1).

Table 1. **The number of polyphenoloxidase (PPO) bands in apple leaves**

Cultivar	Number PPO bands		
	May	July	September
'Noris'	5	5	5
'Papirovka'	4	7	8
'Antonovka'	5	7	8
'Katja'	4	6	6
'Tellissaare'	4	7	5
'Auksis'	3	5	4
'Prima'	3	2	2
'Štaris'	6	8	8
'Aldas'	5	5	5
$LSD_{0.05}$	0.8	1.4	2.3

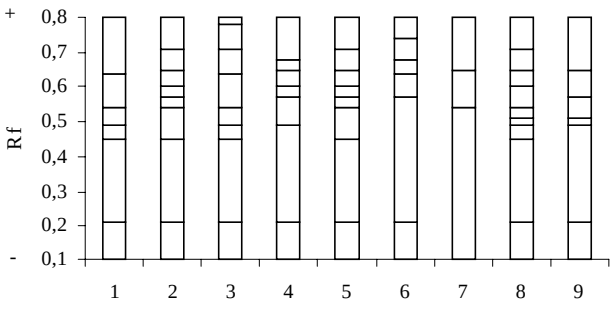

Fig. 1. Pattern of polyphenoloxidase isozyme bands on polyacrylamide gel. PPO extracted from leaves in July. 1 – 'Noris', 2 – 'Papirovka', 3 – 'Antonovka', 4 – 'Katja', 5 – 'Tellissaare', 6 – 'Auksis', 7 – 'Prima', 8 – 'Štaris', 9 – 'Aldas'

The number of PPO bands varied from 2 to 8 in all investigated apple accessions (Fig. 1). A smaller number of PPO bands in May was characteristic of most of the apple cultivars (Table 1). 'Prima' had the highest number of PPO bands (3) in May. The number of PPO bands did not change for 'Noris' and 'Aldas' during the vegetation period and was equal to 5. In 'Papirovka' the highest number of PPO bands was in September (8). 'Katja' and 'Štaris' had the same number of bands in July and September (6 and 8, respectively).

The PPO band at R_f = 0.21 was common for all cultivars except *Prima*. The band at R_f = 0.51 was specific only for the immune apple cultivars and was not detected for the susceptible cultivars.

To confirm scab resistance of the apple cultivars not only at the isozyme but also at the DNA level, PCR using *Vfa1*-specific primers [7] was performed. Our results showed that the cultivars 'Prima', 'Aldas', 'Skaistis' and 'Rudenis', which are immune to 1–5 *Venturia ineaqualis* races, had a DNA fragment 500 bp specific for the *Vfa1* gene (Fig. 2). The immune cultivar 'Štaris' doesn't have this fragment. However, this fragment was detected in the genome of the cultivar 'Tellissare' having a polygenic scab resistance mechanism. The above-mentioned DNA fragment was not found in the susceptible cultivars 'Noris' and 'Papirovka' and the cultivars of polygenic scab resistance 'Katja' and 'Auksis'.

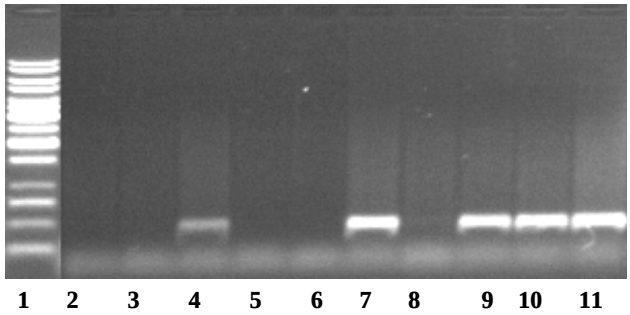

Fig. 2. Electrophoresis of apple DNA fragments amplified by PCR using *Vf1*a specific primer pair. 1 – GeneRuler™ 1kb DNA Ladder, 2 – 'Noris', 3 – 'Papirovka', 4 – 'Tellissare', 5 – 'Auksis', 6 – 'Katja', 7 – 'Prima', 8 – 'Štaris', 9 – 'Aldas', 10 – 'Skaistis', 11 – 'Rudenis'

DISCUSSION

Like an earlier study with peroxidase isozyme [11], our investigations showed that the highest number and well-detectable PPO bands could be obtained at the end of the apple-tree vegetation period. For a polymorphism study in apple cultivars by PPO enzyme systems extracted from expended leaves the best period is from the middle of July to September.

The variability detected for PPO was sufficient to identify accessions by unique banding patterns. A specific band R_f = 0.51 for scab-immune apple cul-

tivars was identified. It seems likely that this band is related to scab resistance. However, this band was characteristic not of all the immune cultivars investigated. Therefore we suppose that the identified band is related to scab resistance, though not reflect all cases of resistance expression. All cultivars having this isoform will be scab-resistant, however, not all immune cultivars will have this isoform. Nevertheless, it shows that using isozyme markers it is possible to distinguish scab-resistant and scab-susceptible cultivars.

DNA investigations show that the above-mentioned isozyme marker is not associated with the *Vfa1* gene. The cultivar 'Štaris', which is not injured by scab so far, has not *Vfa1* gene. Scab resistance in this cultivar is probably governed by an alternative genetic mechanism.

It also been demonstrated that the presence of *Vfa1* does not guarantee scab immunity. This fragment was found in the cultivar 'Tellissaare' possessing a polygenic scab resistance mechanism. Infection of leaves and fruits of this cultivar are estimated by 3 and 1 score points, respectively [12]. In this case a seemingly altered regulation of gene expression takes place. It could be influenced by small changes in gene sequence, caused by strong and steady horizontal selective pressures by the fungal pathogen *V. inaequalis*, and divergent selection on somatic variations [13].

ACKNOWLEDGEMENTS

The authors are grateful to the Lithuanian Ministry of Education and Science for supporting the research programme "Research of plant genetic resources in 2004–2008" and Lithuanian State Science and Studies Foundation for supporting the project No C-03017 "Abiotecha" because this work was carried out within these programmes.

Received 27 April 2005
Accepted 23 July 2005

References

1. Williams EB, Dayton DF, Shay JR. Proc Amer Soc Hort Sci. 1966; 88: 52–6.
2. Æäàíîâ ÂÂ, Ñåäîâ ÅÍ. Ñåëåêöèÿ ÿáëîíè íà óñòîé÷èâîñòü ê ïàðøå. Òóëà, 1991: 207.
3. Chevreau E, Lespinasse Y, Gallet M. Theor Appl Genet 1985; 71: 268–77.
4. Weeden NP, Lamb RC. J Amer Soc Hortic Sci 1985; 110: 509–15.
5. Constabel CP, Yip L, Patton JJ, Christopher ME. Plant Physiology 2000; 124: 285–95.
6. Zawistowski J, Biliaderis CG, Nam E. Robinson DS, Nam E (eds). Oxidative Enzymes in Foods. Elsevier Science Publishers, London, 1991: 217–73.
7. Xu M and Korban SS. Genetics 2002; 162: 1995–2006.
8. Davis BJ. Ann NY Acad Sci 1964; 121: 404–27.
9. Jaaska V. Eesti NSV Tead Akad Toim Biol 1972; 21: 61–9.
10. Dellaporta SL, Wood J, Hicks JB. Plant Mol Biol Rep 1 1983; 19–21.
11. Gelvonauskis B, Ðikðnianienë J. Sodininkystë ir darþininkystë 2001; 20(3)–1: 30–8.
12. Gelvonauskienë D, Bandaravièius A. Sodininkystë ir darþininkystë 1998; 17(1): 30–8.
13. Xu M, Korban SS. Mol Phylogenet Evol 2004; 32(1): 57–65.

D. Gelvonauskienë, J. Ðikðnianienë, R. Rugienius, B. Gelvonauskis, T. Ðikðnianas, V. Stanys, G. Stanienë, A. Sasnauskas, J. Vinskienë

POLIFELOKSIDAZËS IZOFORMOS IR *VFA1* GENO SEKOS SPECIFINIAI ÞYMENYS ÁVAIRAUS ATSPARUMO RAUPLËMS OBELØ VEISLËSE

Santrauka

Siekiant nustatyti izofermentinius þymenis, susijusius su obelø atsparumu rauplëms, tirtas polifenoloksidazës (PFO, EC 1.14.18.1) izoformø spektras. Panaudojant pradmenø porà, specifinæ *Vfa1* geno sekai, atlikta DNR analizë. Nustatyta, kad PFO izoformø kiekis augalø vegetacijos metu keièiasi. Tirtose obelø veislëse stebëtas PFO fermentinës sistemos polimorfizmas. Atskyrus PFO formas poliakrilamido gelyje, identifikuotos specifinës fermento izoformos ar jø kombinacijos, bûdingos rauplëms jautrioms arba atsparioms obelø veislëms. Gauti rezultatai rodo, jog 1–5 raupliø rasei atsparios obelø veislës 'Prima', 'Aldas', 'Skaistis' ir 'Rudenis' turi 500bp DNR fragmentà, kuris yra specifinis *Vfa1* genui. Neserganti rauplëmis veislë 'Ðtaris', kurios atsparumo prigimtis nëra þinoma, minëto fragmento neturi. Ðis fragmentas rastas atsparios rauplëms veislës 'Tellissaare' genome. Jautrios rauplëms obelø veislës 'Noris' ir 'Papirovka' bei poligeninio atsparumo veislës 'Katja', 'Auksis' 500bp DNR fragmento neturëjo.